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Description of *Enterobius* (*Colobenterobius*) *emodensis* sp. n. (Nematoda: Oxyuridae) collected from Central Himalayan langur, *Semnopithecus schistaceus*, in Uttarakhand, India

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Abstract

A new pinworm species, *Enterobius* (*Colobenterobius*) *emodensis* sp. n. (Nematoda: Oxyuridae) is described from the Central Himalayan langur, *Semnopithecus schistaceus*, in Mandal Valley, Chamoli District, Uttarakhand, India, based on mature and immature adults and fourth-stage larvae. This species closely resembles *Enterobius* (*Colobenterobius*) *zakiri* parasitic in Tarai langur, *Semnopithecus hector*, recorded from Uttarakhand and Uttar Pradesh, India, but is readily distinguished by having a shorter esophagus and a shorter spicule. It is surmised that this pinworm has co-speciated with the host langur. The new species is also characterized in that the posterior 1/3 of the esophageal corpus is much darker. Phylogenetic analysis based on the sequences of partial *Cox1* gene of mtDNA suggested a basal position of diversification of *Colobenterobius* from the *Enterobius* lineage.

Key words: Pinworm, Primates, Coevolution, Phylogeny, *Cox1*

Introduction

The langurs of the genus *Semnopithecus* (Mammalia: Primates: Cercopithecidae: Colobinae) are distributed across India and surrounding areas. Although only one species, *Semnopithecus entellus*, was recognized previously, recent studies have demonstrated the presence of seven monotypic species (Wilson & Reeder, 2005). One species, the Central Himalayan langur, *Semnopithecus schistaceus*, is distributed across northwestern Pakistan and the high Himalayan mountains (1,500–4,000 m a.s.l.) of northern India, Nepal and up to the Sankosh River in Bhutan. In China it is distributed in the Tibetan regions of Bo Qu, Ji Long Zang Bu and the Chumbi valleys (IUCN, 2017). During field research of this langur species in the Chamoli District, Uttarakhand, India, we found pinworms discharged in their feces. They belonged to the subgenus *Colobenterobius* of the genus *Enterobius*. From the *Semnopithecus* langurs, two species of pinworms, namely *Enterobius* (*Colobenterobius*) *zakiri* Siddiqi et Mirza, 1954 and *Enterobius* (*Colobenterobius*) *entellus* Hugot, 1987, are known (Siddiqi & Mirza, 1954; Hugot, 1987b). However, close examination revealed that the present worms possess some features differing from those of the previously reported ones as described and discussed herein. DNA sequencing was also performed to elucidate the phylogenetic position of *Colobenterobius*.

Materials and methods

Fecal collection was made for Mandal troop in the Mandal Valley (N 30.4790, E 79.2824; 1,558–2,000m a.s.l.).

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The study troop was comprised of 5 adult males, 13 adult females, 7 subadults and 12 juveniles, during the period from December, 2016 to November, 2017. When pinworms were noticed on the feces with the naked eye during behavioral observation of the troop, several grams of each fecal mass were collected and fixed in 10% formalin and separately in pure ethanol. In the laboratory, each sample was washed with running water on a 100 μm aperture size sieve (Hasegawa, 2009). Residues left on the sieve were transferred to a petri dish and observed under a stereomicroscope to recover the worms. Worms were re-fixed in 70% ethanol, and cleared in glycerol-ethanol solution by evaporation of ethanol. Then, the worms were mounted on glass slides with 50% glycerol for microscopic observation. Figures were made with the aid of a drawing tube. Measurements, mean followed by range, are given in micrometers unless otherwise stated. Type specimens were deposited in the Meguro Parasitological Museum (MPM), Tokyo.

Six worms fixed in pure ethanol were subjected to DNA extraction, amplification and sequencing. DNA extraction from each worm was performed using Quick-DNA™ Miniprep Plus Kit (Zymo Research). The Tks Gflex™ DNA polymerase (TaKaRa) was employed for PCR, together with the manufacturer-supplied reaction buffer. Mitochondrial DNA (mtDNA) of cytochrome *c* oxidase subunit 1 gene (*Cox1*) was targeted with the following original primers: Entero_cox1/F 5'- GTCACCTGATATGAGTTTTCCTCG -3' (forward) and Entero_cox1/R 5'- ACTTAAAACATAATGGAAATGAGC -3' (reverse). The partial 18S ribosomal RNA gene of nuclear DNA (rDNA) was also targeted using primer sets reported by Katayama *et al.* (1993) and Floyd *et al.* (2005): Nem18SF 5'- CGCGAATRGCTCATTACAACAGC -3' (forward) and 18SPCR 5'- ACGGGCGGTGTGTRC -3' (reverse). The original primers N28S/F 5'- TGATTACCCGCTGAACTTAAGCAT -3' (forward) and N28S/R2 5'- TCCTTAGCGGGTACCGACTTCCAT -3' (reverse) were used for amplification of partial 28S rDNA. The PCR was run for 40 cycles (98°C for 10s, 55°C for 30s, and 68°C for 30s) in a total volume of 25 μl including 0.25 μM of each primer and 1 μl of template DNA. The PCR amplicons were sequenced using the BigDye terminator cycle sequencing kit and ABI genetic analyzer 3500 (Applied Biosystems). Each of the PCR primers was used as a sequencing primer. The resultant nucleotide sequences were deposited in the DDBJ/ENA/GenBank databases.

Phylogenetic analysis was made using the neighbor joining method (Saitou & Nei, 1987) and maximum likelihood method for nucleotide and amino acid sequences, respectively, using MEGA6 software (Tamura *et al.*, 2013). *Syphacia frederici*, a murine pinworm from the large Japanese field mouse, *Apodemus speciosus*, was chosen as the outgroup. The bootstrap values were calculated by 1,000 replicates (Felsenstein, 1985).

Results

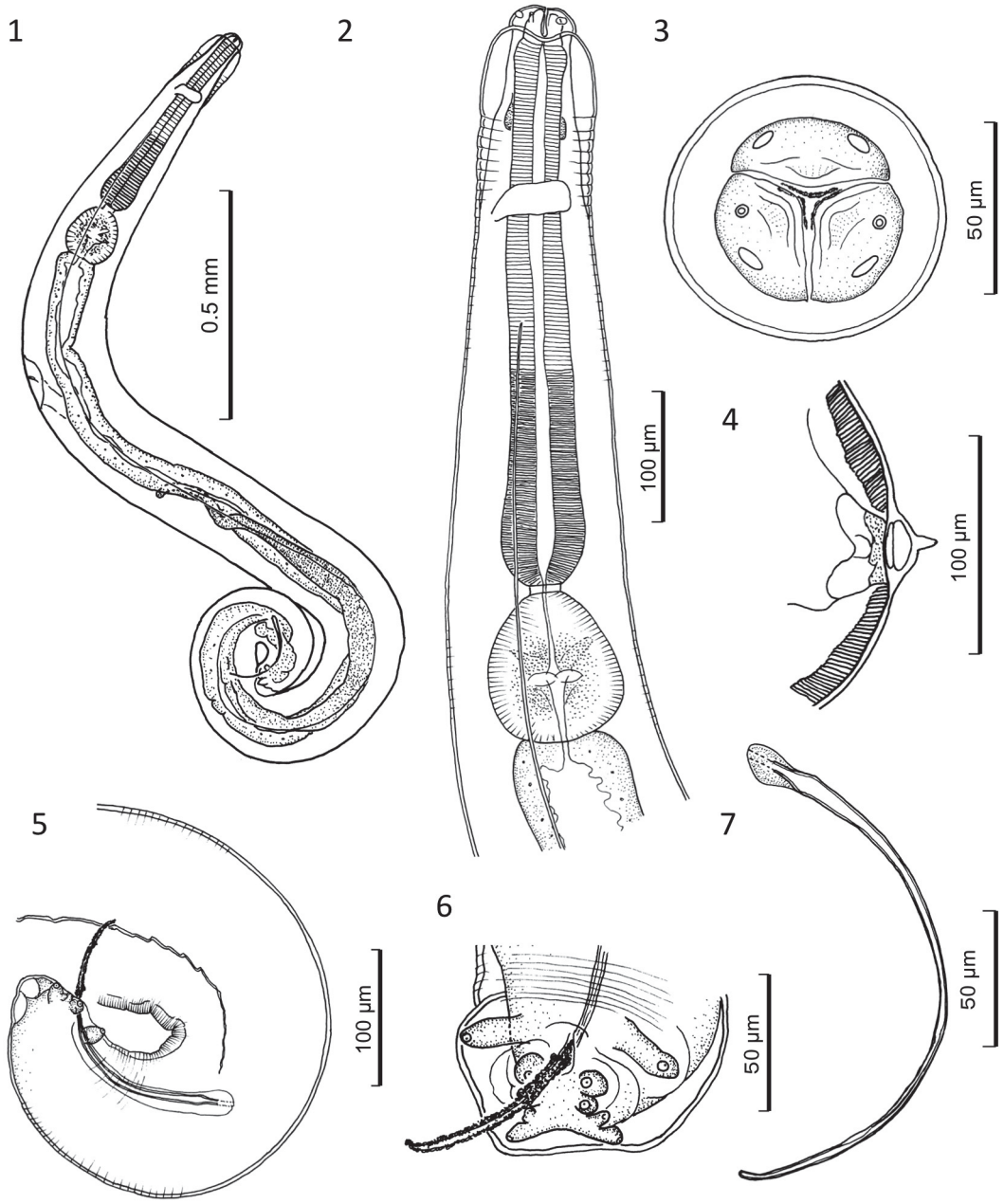
Ten fecal masses were found pinworm positive with the naked eye. In total, 480 male and 233 female pinworms were detected from the feces. The condition of the worms collected from one fecal sample (#37), which was fixed in formalin, was much more suitable for detailed morphological study than those recovered from other feces fixed in ethanol. This sample contained 35 mature males, 30 gravid and 8 immature adult females, and 1 male and 6 female fourth-stage larvae, which were used for the following description. Eleven mature males and 12 gravid females were chosen as type specimen material. Additional measurements of worm length and spicule length were made for 100 males.

Description

***Enterobius* (*Colobenterobius*) *emodensis* sp. n.**
(Figs. 1–22)

General: Body light brown in color. Cephalic expansion well developed, with internal septa in posterior half (Figs. 1, 2, 8). Cuticle with transverse striations. Lateral alae single crested, triangular in cross section (Figs. 4, 12). Cephalic end with 3 developed lips; dorsal lip with 2 cephalic papillae, subventral lips each with cephalic papilla and amphidial pore (Figs. 3, 10). Pharyngeal teeth with lamellated superstructures, corresponding to one-third of lip width in apical view (Figs, 3, 10); slots with cuticular wall extending to the posterior one-fourth of pharynx

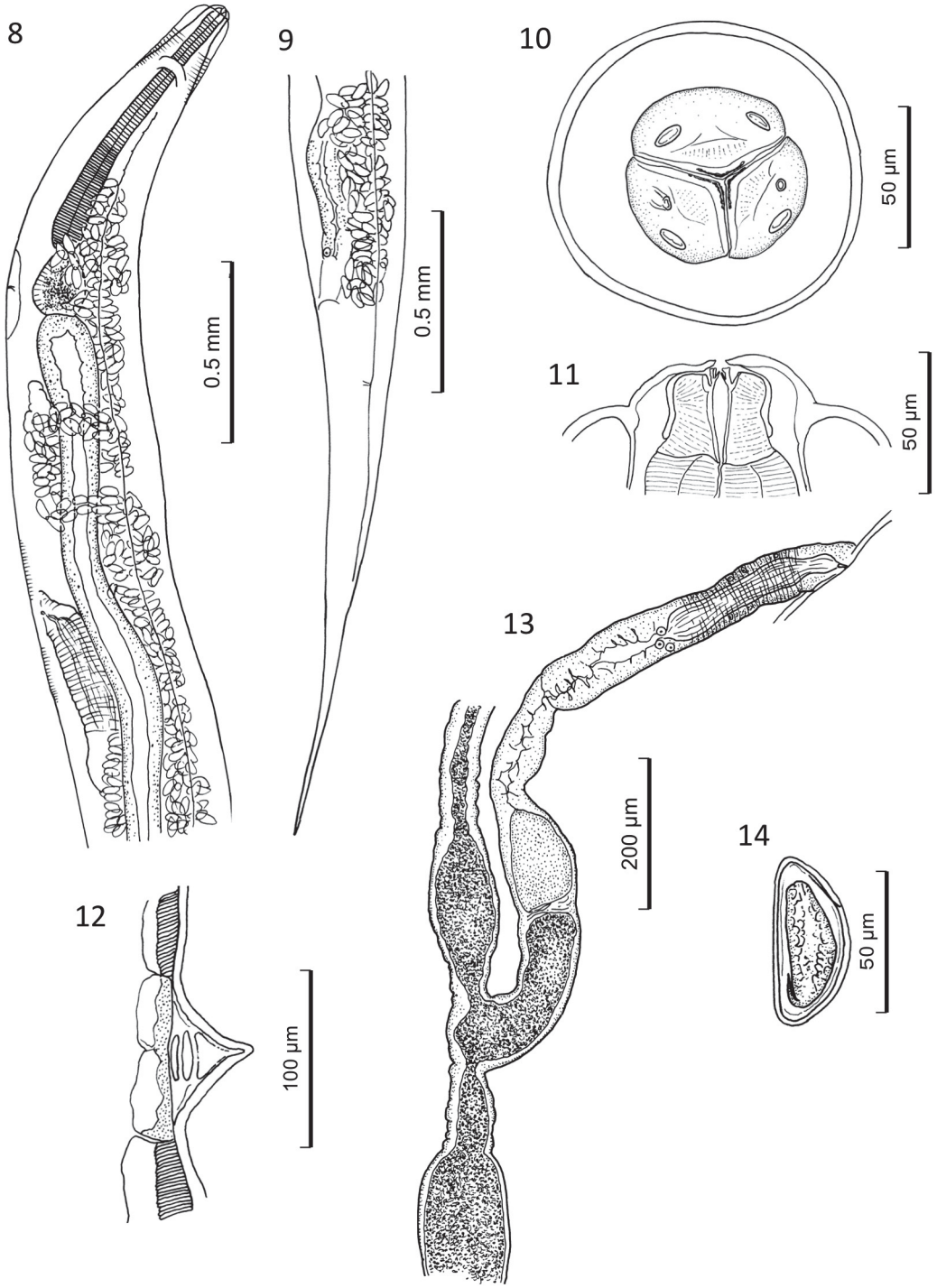
(Fig, 11). Esophageal corpus long club-shaped, with posterior one-third darker containing granules in wall (Figs. 1, 2, 8); short esophageal isthmus present but often hardly discernible in fully gravid females; esophageal bulb valved.



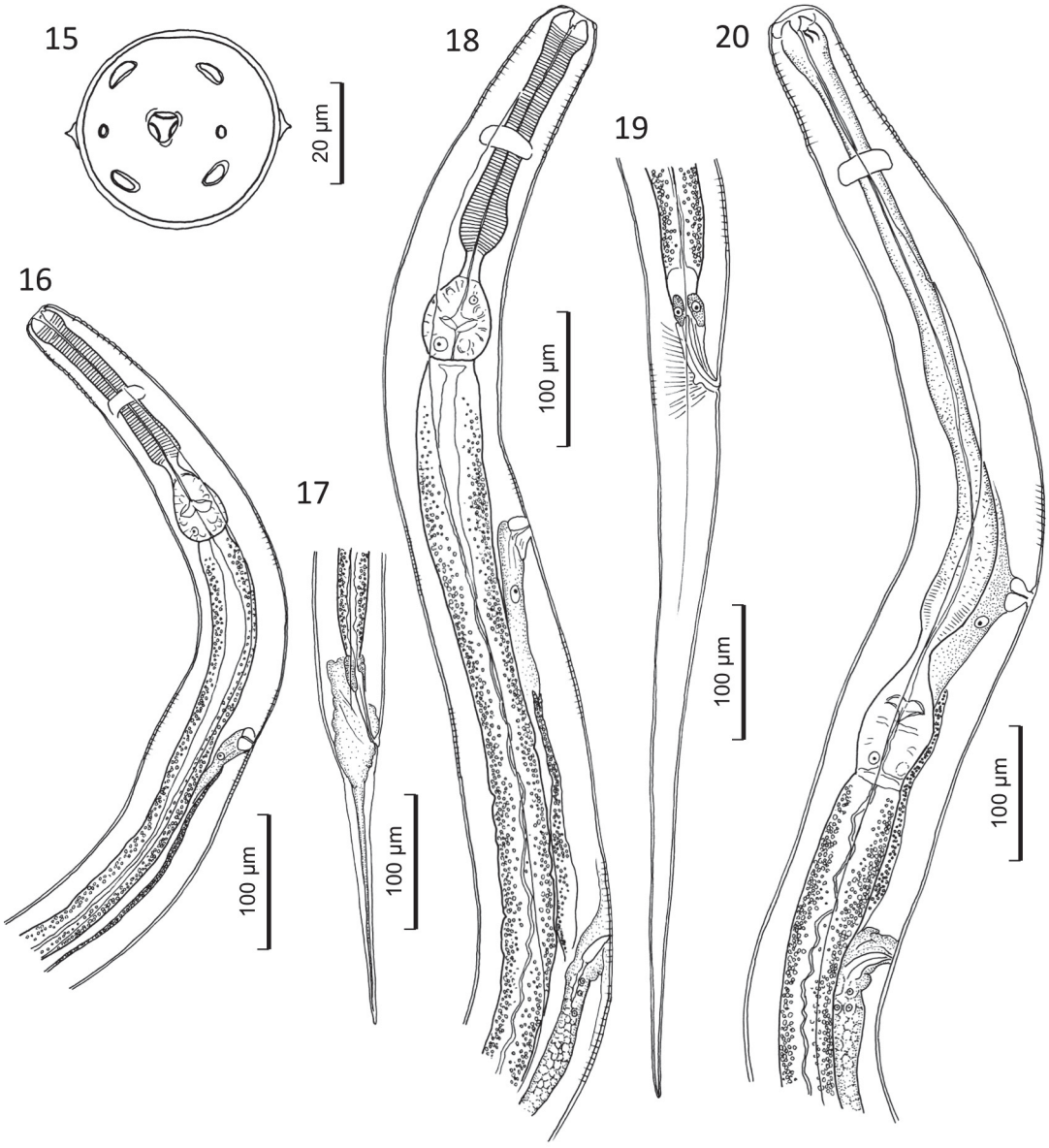
FIGURES 1–7. Male of *Enterobius* (*Colobenterobius*) *emodensis* sp. n. collected from *Semnopithecus schistaceus* in Chamoli District, Uttarakhand, India. 1. Holotype, left lateral view. 2. Enlarged view of anterior portion of holotype, left lateral view. 3. Cephalic end, apical view. 4. Lateral ala in cross section. 5. Posterior portion of holotype, left lateral view. 6. Caudal end, ventral view. 7. Spicule.

Male (Holotype and 10 paratypes): Body tapered to anterior end; posterior body bent ventrally, forming coils (Fig. 1). Length 2.61 (2.01–2.90) mm; maximum width 183 (143–216). Distance between amphidial pores 40. Lateral alae commencing at level slightly posterior to nerve ring, terminating at level anterior to caudal alae (Figs. 2, 5). Cephalic expansion 161 (123–186) long by 89 (75–97) wide. Pharynx 29 (28–32) long; esophageal corpus 431 (355–464) long by 66 (51–79) wide; isthmus 5 (3–6) long by 21 (18–24) wide; bulb 123 (91–135) long by 113 (95–125) wide. Total esophagus length including pharynx, corpus, isthmus and bulb 587 (480–620), corresponding to 22.5 (21.1–24.0) % of worm length. Nerve ring 157 (141–167) and excretory pore 795 (657–976) from cephalic

apex. Caudal alae supported by 4 pairs of papillae: 1st pair precloacal, pedunculate, large, directing laterally; 2nd and 3rd pairs near cloacal aperture, sessile, small, directing ventrally; 4th pair pedunculate, directing posterolaterally; phasmidial pore near base of 4th pair (Figs. 5, 6). Testis directing anteriorly (Fig. 1). Distal portion of testis filled with round spermatozoa each with ca. 2.5 diameter. Spicule slender, 223 (197–238) long, arched, bending ventrally distally, proximal end with light-refractive mass; portion protruding from the cloacal aperture often covered with dark material (Figs. 5–7). Relationship between worm length and spicule length shown in Fig. 23.



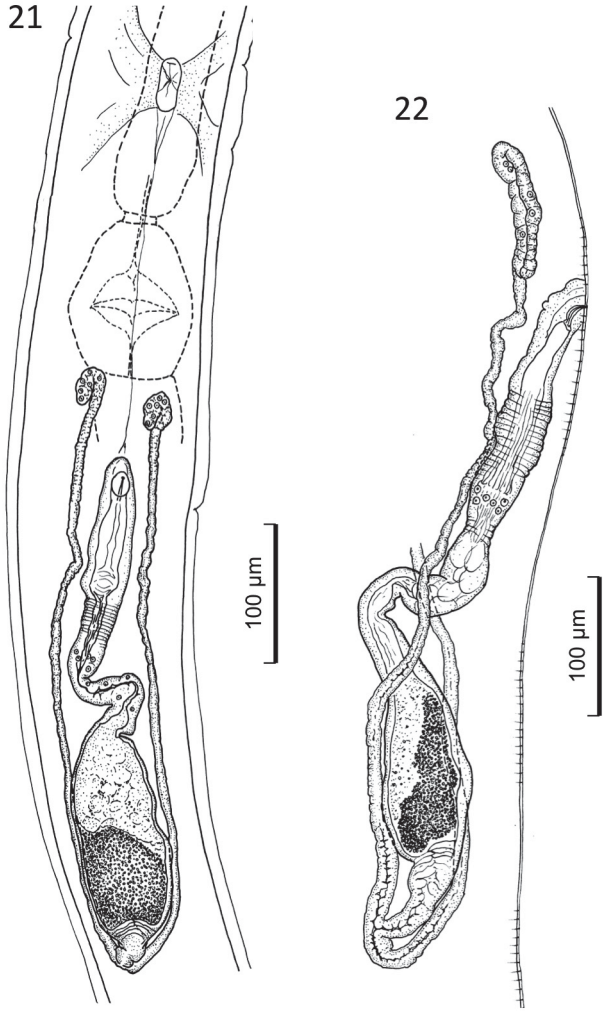
FIGURES 8–14. Female of *Enterobius* (*Colobenterobius*) *emodensis* sp. n. collected from *Semnopithecus schistaceus* in Chamoli, Uttarakhand, India. 8. Anterior portion of allotype, left lateral view. 9. Posterior portion of allotype, left lateral view. 10. Cephalic end, apical view. 11. Cephalic apex sectioned. 12. Lateral ala in cross section. 13. Ovejector, right lateral view. 14. Egg.



FIGURES 15–20. Fourth-stage larvae of *Enterobius* (*Colobenterobius*) *emodensis* sp. n. collected from *Semnopithecus schistaceus* in Chamoli, Uttarakhand, India. 15. Cephalic apex of female, apical view. 16. Anterior portion of male, right lateral view. 17. Posterior portion of male, right lateral view. 18. Anterior portion of female, right lateral view. 19. Posterior portion of female, right lateral view. 20. Anterior portion of premolt female, right lateral view.

Female (Allotype and 11 paratypes): Body tapered to both extremities. Length 7.51 (7.15–8.10) mm, maximum width 428 (360–488). Distance between amphidial pores 42. Lateral alae commencing at level slightly posterior to nerve ring, terminating at middle of tail. Cephalic expansion 243 (204–257) long by 129 (106–143) wide. Pharynx 35 (32–36) long; esophageal corpus 739 (680–776) long by 103 (90–114) wide; isthmus 3 (2–4) long by 26 (24–28) wide (n=5); bulb 168 (162–178) long by 160 (147–172) wide. Total esophagus length 926 (796–992), corresponding to 12.4 (10.7–13.7) % of worm length. Nerve ring 210 (184–249), excretory pore 1.15 (0.94–1.32) mm (n=5) from cephalic apex. Distance from cephalic apex to vulva 1.88 (1.60–2.14) mm, corresponding to 25.0 (21.5–28.7) % of worm length (Fig. 8). Muscular vagina thick, directing posteriorly; vagina uterina with thick epithelium, about twice as long as muscular vagina; cellular wall present dividing length from vagina and opening to uterus ca. 3:1; amphidelphic; uterus of young gravid females filled with dark material, vagina uterina just before cellular wall containing mass of minute round structures each with ca. 2.5 diameter (Fig. 13). Tail slender, gradually tapering to sharp point, 1.49 (1.25–1.73) mm long, occupying 19.8 (16.8–21.6) % of

worm length (Fig. 9). Phasmidial pore opening on lateral ala at level slightly posterior to anus. Eggs with one side flattened, 60.6 ± SD 1.2 (59–63) by 27.1 ± SD 0.6 (26–28) (n=25), convex side with operculum-like structure near pole (Fig. 14).



FIGURES 21, 22. Reproductive organs of immature adult females of *Enterobius* (*Colobenterobius*) *emodensis* sp. n. collected from *Semnopithecus schistaceus* in Chamoli District, Uttarakhand, India. 21. Ventral view (in worm of 2.48 mm long). 22. Right lateral view (in worm of 3.95 mm long).

Fourth-stage larva

General: Cuticle with transverse striations; cephalic portion with wider and prominent annulations (Figs. 16, 18, 20); lateral alae single crested, commencing slightly posterior to cephalic end. Mouth without lips, triangular with rounded corners, surrounded by four cephalic papillae and two amphidial pores (Fig. 15). Esophagus composed of short pharynx, corpus, isthmus and bulb with valve. Tail long, tapering to pointed end (Figs. 17, 19).

Male (1 worm): Length 1.55 mm, maximum width 73. Total esophagus length 212: pharynx 8 long; corpus 137 long by 24 wide; isthmus 16 long by 10 wide; bulb 51 long by 36 wide, corresponding to 13.7 % of worm length. Nerve ring slightly posterior to middle of corpus, 91, excretory pore posterior to esophago-intestinal junction, 364, respectively, from anterior end (Fig. 16). Tail 210 long, corresponding to 13.5 % of worm length; pulp abruptly narrowed posterior to anus (Fig. 17). Spicule and caudal papillae not seen clearly.

Female (2 worms): Length 2.32 (2.28–2.35) mm, maximum width 98 (93–102). Total esophagus length 279

(275–282), corresponding to 12.0 (12.0–12.1) % of worm length: pharynx 18 (16–20) long; corpus 177 (175–178) long by 31 (30–32) wide; isthmus 17 (16–18) long by 13 (12–14) wide; bulb 63 (61–65) long by 50 (48–52) wide. Nerve ring slightly posterior to middle of corpus, 116 (115–117), excretory pore posterior to esophago-intestinal junction, 415 (405–425), primordial vulva 0.70 (0.69–0.73) mm, respectively, from anterior end (Fig. 18). Ovejector directing posteriorly. Tail 0.53 (0.52–0.54) mm long, corresponding to 22.5 (22.1–22.8) % of worm length; pulp not narrowed abruptly (Fig. 19).

Female at premolt and molting phases (4 worms): Length 2.38 (2.20–2.58) mm, maximum width 109 (102–118). Total esophagus length 678 (623–739), corresponding to 28.5 (27.3–30.2) % of worm length: pharynx 31 (26–36) long; corpus elongated, with inconspicuous muscular fibers, 535 (479–574) long by 35 (30–43) wide; isthmus 20 (12–36) long by 17 (14–21) wide, gradually continuous to corpus and bulb; bulb 105 (102–113) long by 72 (58–83) wide. Nerve ring at anterior 1/3 of corpus, 132 (127–137), excretory pore posterior to esophago-intestinal junction, 431 (366–472), and vulva 0.74 (0.70–0.77) mm (n=3), respectively, from anterior end (Fig. 20). Ovejector directing posteriorly; vagina uterina empty. Plug-like structure absent at vulva. Tail 0.53 (0.50–0.57) mm long, corresponding to 22.0 (21.6–24.0) % of worm length; pulp not narrowed abruptly.

Females just after molt (4 worms): Morphology generally identical with those of gravid females except for underdeveloped reproductive organs. Length 2.47 (2.37–2.63) mm, maximum width 127 (118–143). Total esophagus length 689 (656–711), corresponding to 28.0 (26.5–29.7) % of worm length: pharynx 29 (27–32) long; corpus elongated, with muscular fibers and uniform color, 533 (504–552) long by 54 (50–57) wide; isthmus well defined, 7 (6–8) long by 23 (20–28) wide; bulb 118 (111–121) long by 93 (85–97) wide. Nerve ring at anterior 1/3 of corpus, 146 (135–154), excretory pore posterior to esophago-intestinal junction, 438 (385–485), and vulva 0.76 (0.70–0.84) mm (n=3), respectively, from anterior end (Fig. 20). Vagina plugged; ovejector directing posteriorly, vagina uterina containing granulate substance, uterine branches lacking open lumen, running anteriorly to join recurved ovaries (Fig. 21). Tail 0.56 (0.49–0.59) mm long, gradually tapering to sharply pointed end.

Non-gravid female adults (4 worms): Morphology generally identical with females just after molt, but with larger body. Length 4.01 (3.70–4.40) mm, maximum width 211 (196–241). Total esophagus length 790 (759–824), corresponding to 19.7 (18.7–20.5) % of worm length: pharynx 34 (33–36) long; corpus with posterior dark portion, 614 (584–640) long by 76 (75–78) wide; isthmus well defined, 6 (2–8) long by 23 (20–26) wide; bulb 136 (133–143) long by 123 (121–126) wide. Nerve ring 180 (171–192), excretory pore 745 (683–851), and vulva 1.22 (1.13–1.37) mm (n=3), respectively, from anterior end. Vagina plugged; ovejector directing posteriorly, vagina uterina containing mass of round structures each with 2.5 diameter, uterine branches elongated but still lacking open lumen; ovaries recurrent posteriorly (Fig. 22). Tail 0.83 (0.67–0.96) mm long.

Taxonomic summary

Type host: Central Himalayan langur, *Semnopithecus schistaceus* (Primates: Cercopithecidae: Colobinae)

Site in host: Intestine (Discharged in feces).

Type locality: Siroli Village, District- Chamoli, State-Uttarakhand, India.

GPS coordinates: Latitude: N 30.467526, Longitude: E79.275426, Altitude: 1,844 m a.s.l.

Date of collection: 17 June 2017.

Specimens deposited: MPM Coll. No. 21455 (Holotype male and allotype female); No. 21456 (10 male and 11 female paratypes); No. 21457 (fourth-stage larvae, molting larvae and postmolt juveniles; 15 worms).

Etymology. Species epithet was chosen after the Himalayan distribution of the pinworm.

Remarks. By having an esophagus with posterior bulb with valve, reduced number of caudal papillae, only one spicule and no precloacal sucker in male, eggs with one side flattened, the present species belongs to the order Oxyurida, which contains only superfamily Oxyuroidea (Chabaud, 1974). By having non-pedunculate amphids, and the genital cone of male lacking a sclerotized supporting structure, it belongs to the family Oxyuridae (Petter & Quentin, 1976). Presence of a cellular wall (so-called 'diaphragm') in the ovejector along with other morphological characters and host preference, assigns it to the subfamily Enterobiinae (Hugot *et al.*, 1996). This subfamily includes the genera *Enterobius*, *Lemuricola*, *Trypanoxyuris* and *Xeroxyuris*. By lacking a caudal appendix and having an amphidelphic genital tract, it belongs to the genus *Enterobius*. By having pharyngeal teeth with superstructures, the present species is placed in the subgenus *Colobenterobius* (Hugot *et al.*, 1996). Twelve species

are known in this subgenus: *E*. (*C*.) *colobis* Vuylstéke, 1964, *E*. (*C*.) *guerezae* Hugot, 1987, *E*. (*C*.) *inglisi* Wahid, 1961, *E*. (*C*.) *paraguerezae* Hugot, 1987 and *E*. (*C*.) *pesteri* Wahid, 1961 from African colobuses, and *E*. (*C*.) *entellus* Hugot, 1987, *E*. (*C*.) *longispiculum* Quentin *et al.*, 1979, *E*. (*C*.) *pitheci* Cameron, 1929, *E*. (*C*.) *presbytis* Yin, 1973, *E*. (*C*.) *pygatrichus* Hasegawa *et al.*, 2002, *E*. (*C*.) *serratus* Hasegawa *et al.*, 2003 and *E*. (*C*.) *zakiri* Siddiqi et Mirza, 1954, from oriental colobuses (see Hugot, 1987a, b; Hasegawa *et al.*, 2002, 2003).

The present species is easily distinguished from the African representatives, namely, *E*. (*C*.) *colobis*, *E*. (*C*.) *guerezae*, *E*. (*C*.) *paraguerezae*, *E*. (*C*.) *pesteri*, all of which have large pharyngeal teeth, (Vuylstéke, 1964; Wahid, 1961; Hugot, 1987a; Hasegawa *et al.*, 2008). The pharyngeal teeth have not been understood adequately in *E*. (*C*.) *inglisi*, which was described based on only two males. However, *E*. (*C*.) *inglisi* could be distinguished from the present species by having a longer spicule (0.3 mm long in males with body length of 3 to 3.2 mm) with pointed tip (Wahid, 1961). Among the oriental members of the subgenus, both sexes have been described in *E*. (*C*.) *entellus*, *E*. (*C*.) *longispiculum*, *E*. (*C*.) *presbytis* and *E*. (*C*.) *zakiri*, while only females have been known in *E*. (*C*.) *pitheci*, *E*. (*C*.) *pygatrichus* and *E*. (*C*.) *serratus*. By having wide pharyngeal superstructures, corresponding to about 1/3 of lip width, the present species differs from *E*. (*C*.) *entellus*, *E*. (*C*.) *presbytis* and *E*. (*C*.) *pygatrichus*, which have much narrower pharyngeal superstructures (Quentin *et al.*, 1979; Hugot, 1987b; Hasegawa *et al.*, 2002, 2003). Distinguishing characters other than the width of pharyngeal superstructures are as follows: *E. (C.) entellus* has a much shorter spicule (100 µm long) and smaller ratio of total esophagus length to worm length in males (15%) (Hugot, 1987b); *E.* (*C.*) *presbytis* possesses a much shorter esophagus in females (corresponding to 6.6 to 8.3 % of worm length; Hugot, 1987b); and *E.* (*C.*) *pygatrichus* has a much shorter esophagus and a shorter tail in females (corresponding 7.0 to 7.9% and 9 to 12.7 %, respectively, of worm length; Hasegawa *et al.*, 2002).

By having wide pharyngeal superstructures, the present species resembles *E*. (*C*.) *longispiculum*, *E*. (*C*.) *pitheci*, *E*. (*C*.) *serratus* and *E*. (*C*.) *zakiri* (Quentin *et al.*, 1979; Hugot, 1987b; Hasegawa *et al.*, 2003). However, it is readily distinguished from the former three species by the following features: *E*. (*C*.) *longispiculum* has a relatively shorter esophagus in males (corresponding to 14.2% of worm length), and smaller eggs (52 by 27.5 µm) (Quentin *et al.*, 1979); *E*. (*C*.) *pitheci* has a relatively shorter esophagus (corresponding to 9.2 to 9.7 % of worm length: Cameron, 1929; Hugot, 1987b) and a shorter tail (occupying 13.7% or 14.3% of worm length) in females and smaller eggs (50 to 55 by 25 µm) (Hugot, 1987b); *E*. (*C*.) *serratus* has serrated inner margins of lips (Hasegawa *et al.*, 2003); and *E*. (*C*.) *zakiri* has a longer spicule (350 µm) in males (Siddiqi & Mirza, 1954; Hugot, 1987b).

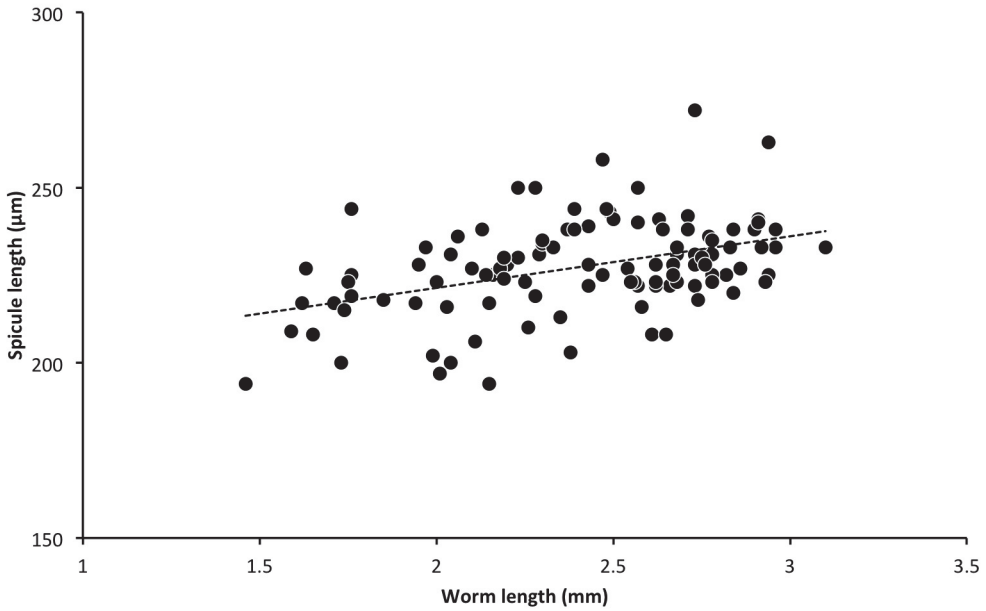


FIGURE 23. Relationship of spicule length to worm length in males (Approximation formula: y = 14.725x + 191.94; n=112).

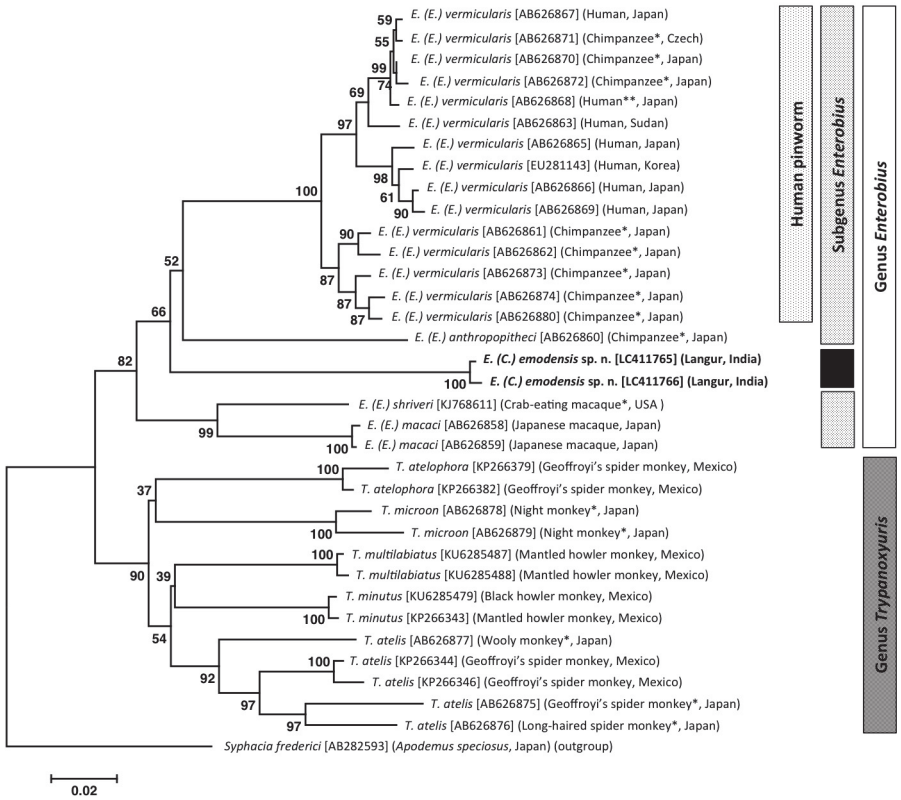


FIGURE 24. Phylogenetic reconstruction of primate pinworms based on partial *Cox1* nucleotide sequences each with 599 positions using Neighbor-joining method. Asterisks indicate captive individuals.

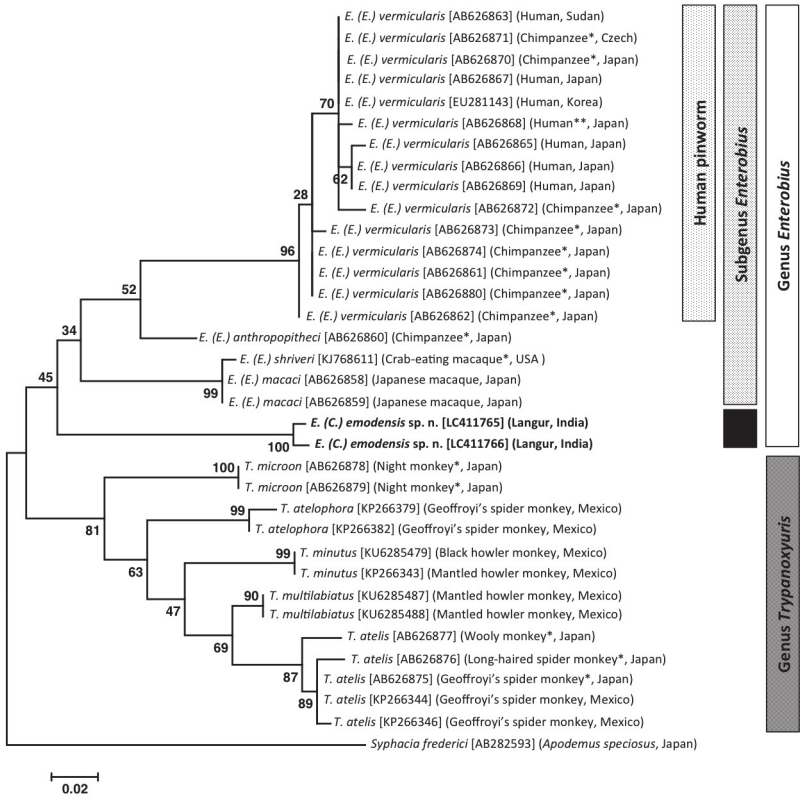


FIGURE 25. Phylogenetic reconstruction of primate pinworms based on partial *Cox1* amino acid sequences each with 199 positions using Maximum Likelihood method. Asterisks indicate captive individuals.

Phylogenetic position by DNA sequencing. DNA amplification was successful only for two individuals out of six worms tested with the *Cox1* primers. Meanwhile, none of the worms responded positively to the 18S rDNA and 28S rDNA primers. Two sequences of partial *Cox1* (each 851 bp) from the two worms (LC411765 and LC411766) had T-C and G-C substitutions at two sites, which resulted in two amino acid substitutions, M-T and W-C. Phylogenetic analysis was made on 599 nucleotides and 199 amino acid sequences, which overlapped with those of primate pinworms already deposited in the DNA database. The Kimura 2-parameter model (Kimura, 1980) was chosen for Neighbor Joining reconstruction on nucleotide sequences and the JTT+G model was applied for Maximum Likelihood analysis for amino acid sequences. Representative sequences of partial *Cox1* of *Enterobius* and *Trypanoxyuris* pinworms from both Old and New World primates and humans were also analyzed with the present sequences.

Figures 24 and 25 show phylogenetic trees reconstructed based on nucleotide and amino acid sequences, respectively. In the amino acid-based tree, outgroup setting was not necessary because *S. frederici* was located most basally. In both trees, lineages to the Old World genus *Enterobius* and the New World genus *Trypanoxyuris* were first divided. In the Old World lineage of the nucleotide-based tree, the common ancestor of macaque pinworms (*Enterobius macaci* and *Enterobius shriveri*) first diverged and then the lineage to the present species separated, leaving chimpanzee pinworm (*Enterobius anthropopitheci*) and human pinworm (*Enterobius vermicularis*) to diversify subsequently. In the tree based on amino acid sequences, general topology was similar with that of the nucleotide-based one. However, *E.* (*C.*) *emodensis* was first diverged from the common ancestor of other Old World representatives. In both nucleotide and amino acid-based trees, the bootstrap values at nodes of early diversification in Old World lineage were generally low.

Discussion

It has been generally considered that pinworms have coevolutionary relationships with their vertebrate hosts often showing specificity to host genus (see Cameron, 1929; Brooks & Glen, 1982; Hugot, 1999). The subgenus *Colobenterobius* is considered to have coevolved with colobine monkeys (Hugot, 1999). According to the phylogenetic relationship of Old World primates, the common ancestor of colobines and macaques diverged from the lineage to Hominoidea in the early Oligocene, about 25 Mya BP, and then diverged into colobines, macaques, and other monkeys (Wilkinson, *et al.* 2011; Springer, *et al.* 2012; Stevens, et al 2013). The basal position of the divergence of macaque and langur-parasitic pinworms from the lineage to the hominioid-parasitic species shown in the *Cox1* phylogeny may coincide with this evolutionary history of host primates. This assumption could be further supported by rDNA sequencing in the future.

It is of special interest that some colobine genera host more than one species of *Colobenterobius*: *Trachypithecus* harbors three pinworm species, namely *E.* (*C.*) *longispiculum* (from *T. obscurus* of Malay), *E.* (*C.*) *pitheci* (from *T. pileatus* of Assam) and *E.* (*C.*) *presbytis* (from *T. phayrei* of Yunnan and *T. cristatus* of Malay); *Semnopithecus* also harbors three pinworm species, namely *E.* (*C.*) *entellus* and *E.* (*C.*) *zakiri* from *S. entellus* (Siddiqi & Mirza, 1954; Quentin *et al.*, 1979; Hugot, 1987b; but see discussion on the host species below) and the present species from *S. schistaceus*. The presence of more than one species suggests rapid speciation of pinworms in *Trachypithecus* and *Semnopithecus*, which are considered to be close together phylogenetically (Osterholtz *et al.*, 2008).

The type locality of *E.* (*C.*) *zakiri* was Garhwal, Uttarakhand (Siddiqi & Mirza, 1954). This species was later found from *S. entellus* in Allahabad, Uttar Pradesh, India, and also collected as a mixed infection with *E. (C.) entellus* from the same host species autopsied at the zoological garden in Giza, Egypt (Hugot, 1987b). The *Semnopithecus* langurs in Northern India were previously assigned to a single species, *S. entellus*, but today is regarded to be separated into three species, *S. schistaceus*, *S. hector* and *S. ajax* (see Ashalakshmi et al, 2014). Siddiqi & Mirza (1954) recorded the host as *S. entellus schistaceus* but called it the 'Tarai langur', which is today assigned to *S. hector*. Hence, it is strongly surmised that the actual type host of *E. (C.) zakiri* was *S. hector*. If this is the case, it can be suggested that the pinworms have co-speciated with the host langurs. Further study employing DNA sequence analysis along with morphological and ecological analyses is also expected to elucidate the evolutionary process of *Colobenterobius* in the Indian colobines.

Observation of the fourth-stage female larvae revealed that the esophagus is rapidly elongated more than twice during the perimolt period. This elongation of the esophagus shifts the position of the excretory pore from the post-

to pre-esophago-intestinal junction level. In mature females, the excretory pore opens near the level of the esophago-intestinal junction apparently due to extension of cuticle of the anterior body. The isthmus in the fourth-stage larvae is smoothly continuous to the corpus and bulb, but becomes a distinct structure during the perimolt period. Sex differences are seen in tail morphology and length in the fourth-stage. Because adult males lack the tail process, it is suggested that the pulp of the male tail is rapidly absorbed during the premolt phase.

It is noteworthy that all immature females after molt had a mass of round structures in their vagina uterina. These structures are considered to be spermatozoa because the shape and diameter of these structures are the same as the spermatozoa found in the testis. It is hence suggested that copulation was made immediately after the molt. Such an early copulation of immature female pinworms with adult males has been well documented in Oxyurida (see Hugot *et al.*, 1996). Moreover, the plug-like structure/substance at the vulva has been described/drawn for various pinworms (e.g. Hugot, 1984, 1986; Hasegawa *et al.*, 2002, 2008; Kuze *et al.*, 2010). It may act as a copulation plug, preventing further copulation with other males.

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